Plasmodium cynomolgi Co-infections among Symptomatic Malaria Patients, Thailand

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Among 1,180 symptomatic malaria patients, 9 (0.76%) infected with *Plasmodium cynomolgi* were co-infected with *P. vivax* (n = 7), *P. falciparum* (n = 1), or *P. vivax* and *P. knowlesi* (n = 1). Patients were from Tak, Chanthaburi, Ubon Ratchathani, Yala, and Narathiwat Provinces, suggesting *P. cynomolgi* is widespread in this country.

Plasmodium cynomolgi, a simian malaria parasite, possesses biological and genetic characteristics akin to those of the most widespread human malaria parasite, *P. vivax*. Although *P. cynomolgi* circulates among monkey species such as long-tailed macaques (*Macaca fascicularis*) and pig-tailed macaques (*M. nemestrina*), experimental and accidental transmissions have been implicated in symptomatic infections in humans (*1*). Several mosquito vectors for human malaria can also transmit *P. cynomolgi*, posing the risk of cross-species transmission in areas where its natural hosts coexist with people (*1*,*2*). Among pig-tailed and long-tailed macaques living in various countries in Southeast Asia, including Thailand, *P. cynomolgi* infections are not uncommon (*3*,*4*). A case of naturally transmitted *P. cynomolgi* malaria in a human was reported from eastern Malaysia (*5*). Subsequent surveillance in western Cambodia and northern Sabah state in Malaysia revealed asymptomatic human infection, albeit at low prevalence (*6*,*7*). Symptomatic *P. cynomolgi* infection was diagnosed in a traveler returning to Denmark from Southeast Asia (*8*). During testing of symptomatic malaria patients in Thailand, we identified 9 co-infected with cryptic *P. cynomolgi* and other *Plasmodium* species.

The Study

We examined 1,359 blood samples taken from febrile patients who sought treatment at malaria clinics or local hospitals in 5 Thailand provinces: Tak (n = 192, during 2007–2013), Ubon Ratchathani (n = 239, during 2014–2016), Chanthaburi (n = 144, during 2009), Yala (n = 592, during 2008–2018), and Narathiwat (n = 192, during 2008–2010). Using microscopy, we found 1,152 cases in which malaria was caused by *P. vivax* (869 patients, 75.43%), *P. falciparum* (272 patients, 23.61%), or co-infection with both species (11 patients, 0.96%). Using species-specific nested PCR, including for *P. cynomolgi* (Appendix, https://wwwnc.cdc.gov/EID/article/27/2/19-1660-App1.pdf), targeting the mitochondrial cytochrome *b* gene (*mtCytb*) of 5 human malaria species for molecular detection, as described elsewhere (*9*,*10*), we found malaria in 1,180 patients; *P. vivax* infections exceeded *P. falciparum* infections (Table 1). Submicroscopic parasitemia occurred in 28/1,180 (2.4%) patients: 19 infected with *P. vivax*, 7 with *P. falciparum*, 1 with *P. vivax* and *P. falciparum*, and 1 with *P. malariae*.

The mean age of all patients was 26.3 (range 7–85) years; 940/1,180 (79.7%) of patients were men. Febrile symptoms, lasting 1–7 days (mean 3.1, SD ±1.3 days) before blood sample collection, developed in all PCR-positive malaria patients. Monoinfection with *P. knowlesi* occurred in 4 patients, *P. malariae* in 3, and *P. ovale* in 1. We detected co-infections in 77 (0.93%) patients; of these co-infections, 55 were *P. falciparum* and *P. vivax*. In total (i.e., including both monoinfections and co-infections), *P. knowlesi* was detected in 18 patients, of which 10 cases were newly identified from Ubon Ratchathani Province, which borders Cambodia and Laos.

We detected *P. cynomolgi* in 9 patients, all of whom were co-infected with *P. vivax* (n = 7), *P. falciparum* (n = 1), or both *P. vivax* and *P. knowlesi* (n = 1). The overall prevalence of *P. cynomolgi* infections was 0.76%. Patients infected with *P. cynomolgi* were found in all provinces. Although 5 of these patients were from Yala Province, the proportion of *P. cynomolgi* infections among malaria cases in each malaria-endemic area (0.52%–0.87%) was comparable.

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DOI: https://doi.org/10.3201/eid2702.191660

Table 1. Distribution of *Plasmodium* infections diagnosed by PCR of blood samples taken from febrile patients who sought treatment at malaria clinics or local hospitals in 5 provinces, Thailand*

Species	No. cases by province					Total no. cases	% Tota cases
	Tak	Ubon Ratchathani	Chanthaburi	Yala	Narathiwat		
P. vivax	98	57	141	467	59	822	69.66
P. falciparum	72	41	0	87	73	273	23.14
P. knowlesi	0	4	0	0	0	4	0.34
P. malariae	0	2	0	1	0	3	0.25
P. ovale	0	0	0	1	0	1	0.09
P. vivax + *P. falciparum*	21	8	0	11	15	55	4.66
P. vivax + *P. knowlesi*	0	3	2	0	4	9	0.76
P. vivax + *P. cynomolgi*	1	1	1	3	1	7	0.59
P. vivax + *P. knowlesi* + *P. cynomolgi*	0	0	0	1	0	1	0.09
P. falciparum + *P. knowlesi*	0	3	0	1	0	4	0.34
P. falciparum + *P. cynomolgi*	0	0	0	1	0	1	0.09
PCR-positive	192	119	144	573	152	1,180	100.00
PCR-negative	0	120	0	19	40	179	NA
Total no. samples tested	192	239	144	592	192	1,359	NA

*NA, not applicable.

DNA from 10 *P. knowlesi* isolates from Ubon Ratchathani Province and the 9 *P. cynomolgi* isolates were subject to nested PCR amplification spanning a 1,318-bp region of mitochondrially encoded cytochrome c oxidase I (*mtCOX1*). Direct sequencing of the purified PCR-amplified template was successfully performed from all 10 *P. knowlesi* and from 6 *P. cynomolgi* isolates. The remaining 3 *P. cynomolgi* isolates could not be further amplified due to inadequate DNA in the samples. All *mtCOX1* sequences of *P. knowlesi* from Ubon Ratchathani Province were different from one another and distinct from those from the previous case of natural human infection in Thailand (GenBank accession no. AY598141) (*11*). All 6 amplified *P. cynomolgi* isolates contained different sequences belonging to 2 clades. One was closely related to the Gombak strain (accession no. AB444129) and the remaining 5 isolates were clustered with the RO strain (accession no. AB444126) (Figure 1).

All but 1 *P. cynomolgi* infection occurred in male patients (age 15–53 years, median 32 years). Most *P. cynomolgi* malaria patients resided in areas where domesticated or wild macaques were living in proximity to humans. Infections with *P. cynomolgi* occurred in different annual periods; more cases were detected in rainy seasons than in dry seasons (Table 2). The parasite density of *P. cynomolgi* could not be determined from blood smears because of morphologic resemblance to *P. vivax*; an isolate co-infected with *P. falciparum* (YL3634) had very low parasitemia. Of 8 patients with *P. cynomolgi* co-infection, 6 had parasitemia <10,000 parasites/μL (<0.2% parasitemia). It remains unknown whether *P. cynomolgi* was co-responsible for symptomatic infections or merely coexisted asymptomatically with other human malaria parasites. However, self-reported defervescence among *P. cynomolgi*–co-infected patients occurred 1–3 days after antimalarial treatment with chloroquine plus primaquine after onsite microscopic diagnosis of *P. vivax* malaria or artesunate plus mefloquine for *P. falciparum* malaria. Unfortunately, data on long-term follow-up were not available.

Conclusions

This report highlights the presence of *P. cynomolgi* in the human population of Thailand, where natural hosts, both pig-tailed and long-tailed macaques, are prevalent. All patients with *P. cynomolgi* infections harbored either *P. falciparum* or *P. vivax* in their blood, implying that this simian malaria species could share the same anopheline vectors or have different vectors with similar anthropophilic and zoophilic tendencies. The presence of *P. cynomolgi* in diverse malaria-endemic areas of Thailand suggests that cross-species transmission has occurred. Human infection with *P. cynomolgi* seems not to be newly emerging because it was detected among blood samples collected over a range of time periods since 2007. Undoubtedly, morphologic similarity between *P. cynomolgi* and *P. vivax* can hamper conventional microscopic diagnosis (*1*,*5*,*8*). Cryptic co-existence of simian and human malaria species could further preclude accurate molecular detection when inadequate diagnostic devices are used.

Previous surveys of *Plasmodium* infections in pig-tailed and long-tailed macaques have revealed the presence of *P. cynomolgi* and other simian malaria species in Thailand, mainly in the southern part of the country (*4*). Most patients infected with *P. cynomolgi* resided in areas where macaques were living in proximity to humans; therefore, the risk of acquiring malaria from this parasite could increase as people encroach into the habitats of infected macaques, as happened with malaria caused by *P. knowlesi*. Of note,

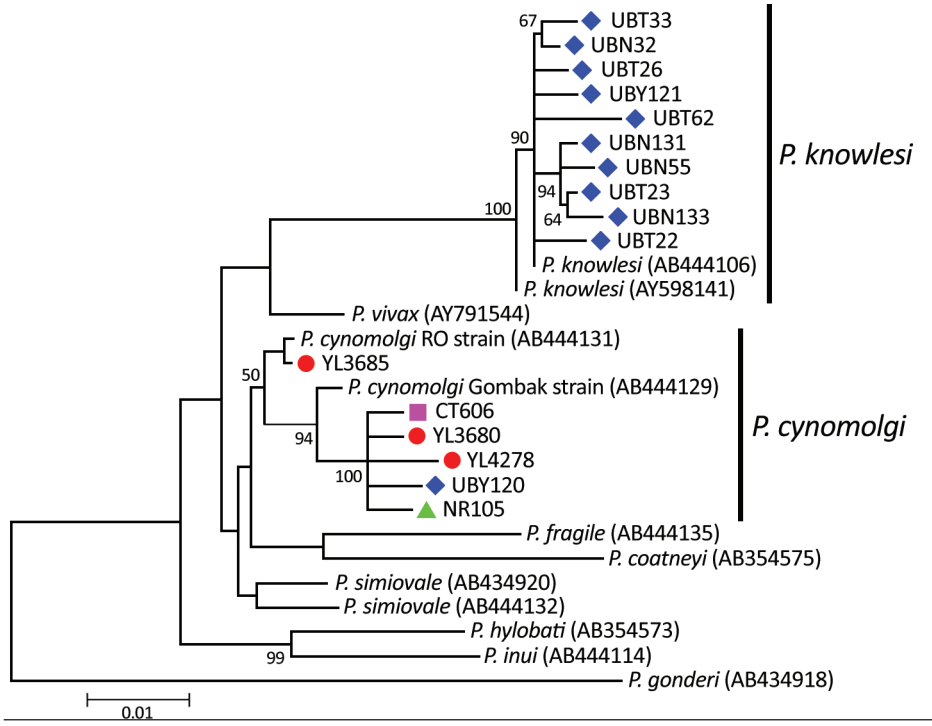

Figure. Maximum-likelihood phylogenetic tree inferred from mitochondrially encoded cytochrome *c* oxidase I of *Plasmodium cynomolgi* and *P. knowlesi* from Thailand compared with other closely related species. Tree spans 1,318-bp region. Colors indicate province where human isolates were found: red circles, Yala; green triangle, Narathiwat; purple square, Chanthaburi; and blue diamonds, Ubon Ratchathani. GenBank accession numbers of reference sequences are given in parentheses. Bootstrap values >50% based on 1,000 pseudoreplicates are shown on the branches. Scale bar indicates nucleotide substitution per site.

co-infection with *P. cynomolgi*, *P. knowlesi*, and *P. vivax* occurred in a patient in Yala Province whose housing area was surrounded by several domesticated pig-tailed and long-tailed macaques.

Analysis of the *mtCOX1* sequences of *P. cynomolgi* among 6 patients showed that all isolates possessed different genetic sequences, suggesting that several strains or clones of this simian parasite are capable of cross-transmission from macaques to humans. Meanwhile, *P. cynomolgi* seems to contain 2 divergent lineages (*12*), represented by RO and Gombak strains. The *mtCOX1* sequences of both *P. cynomolgi* lineages were found in human-derived isolates in this study, further supporting that diverse strains of this parasite can infect people. Likewise, sequence diversity in the *mtCOX1* of *P. knowlesi* from Ubon Ratchathani Province suggests that cross-transmission from macaques to humans may not be restricted to particular parasite strains.

Table 2. Demographic and parasitologic features of *Plasmodium cynomolgi*–co-infected patients among febrile patients who sought treatment at malaria clinics or local hospitals in 5 provinces, Thailand

Patient*	Age, y/sex	Province	Month	Season	Monkey in proximity	Microscopy diagnosis	Parasites/μL‡	PCR diagnosis
TSY1522	38/M	Tak	2007 Nov	Dry	No	*P. vivax*	12,160	*P. vivax*, *P. cynomolgi*
CT606†	30/M	Chanthaburi	2009 Oct	Rainy	Yes	*P. vivax*	86,535	*P. vivax*, *P. cynomolgi*
UBY120	32/M	Ubon Ratchathani	2015 Aug	Rainy	Yes	*P. vivax*	570	*P. vivax*, *P. cynomolgi*
NR105	53/M	Narathiwat	2008 Jul	Rainy	Yes	*P. vivax*	4,620	*P. vivax*, *P. cynomolgi*
YL3179	15/M	Yala	2016 Apr	Dry	Yes	*P. vivax*	1,140	*P. vivax*, *P. knowlesi* *P. cynomolgi*
YL3634	40/F	Yala	2016 Dec	Rainy	Yes	*P. falciparum*	60	*P. falciparum*, *P. cynomolgi*
YL3680	49/M	Yala	2016 Dec	Rainy	Yes	*P. vivax*	3,720	*P. vivax*, *P. cynomolgi*
YL3685	18/M	Yala	2016 Dec	Rainy	Yes	*P. vivax*	4,680	*P. vivax*, *P. cynomolgi*
YL4278	21/M	Yala	2017 Oct	Rainy	Yes	*P. vivax*	7,440	*P. vivax*, *P. cynomolgi*

*Alphanumeric designations represent provinces and serial number of blood samples.
†Patient from Cambodia, but had lived in Thailand for 1 year just prior to illness, with no history of travel outside of the country.
‡All species of malaria parasites (all stages) were determined from ≥200 leukocytes on Giemsa-stained thick blood films.

Although human malaria from either parasite may be asymptomatic, infection with *P. knowlesi* can result in death, but patients infected with *P. cynomolgi* at worst had only benign symptoms (*5*–*8*). However, severe and complicated malaria has been observed in rhesus macaques experimentally infected with *P. cynomolgi* (*13*).

Whether severe cynomolgi malaria can occur in humans remains to be elucidated. However, if human infections with *P. cynomolgi* do become public health problems, diagnostic and control measures might be complicated by the morphological similarity between *P. vivax* and *P. cynomolgi*. This possibility makes further surveillance of this simian malaria in humans mandatory.

Acknowledgments

We are grateful to all patients who provided blood samples and to staff at local malaria clinics and hospitals for assistance in field studies.

Funding for this study was provided to C.P. by the Thailand Research Fund (grant no. RSA5980054), to C.P. by the Asahi Glass Foundation (in fiscal year 2016), and to C.P. and S.J. by the Thai Government Research Budget for Chulalongkorn University (grant nos. GRB-APS-12593011 and GBA-600093004).

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Article DOI: https://doi.org/10.3201/eid2702.191660

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Appendix

We performed primary DNA amplification in a 30 μL reaction mixture containing template DNA, 2.5 mmol/L $MgCl_2$, 300 mmol/L each deoxynucleoside triphosphate, 3 μL of 10X ExTaq PCR buffer (Takara Bio; https://www.takara-bio.com), 0.3 μmol/L of primers PCOX1-F0 and PCOX1-R0, and 1.25 units of ExTaq DNA polymerase (Takara Bio). The thermal cycle profile contained preamplification denaturation at 94°C for 1 min followed by 35 cycles of 94°C for 40 s; 50°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. We performed secondary PCR in 2 separate reactions in a total volume of 30 μL, one using primers Pcy1COX1-F and PcyCOX1-R and the other using primers Pcy2COX1-F and PcyCOX1-R. The reaction mixtures were essentially the same as those for primary PCR except for the primers and 2 μL of primary PCR product as templates. We performed all amplifications in an Applied Biosystem GeneAmp PCR System 9700 thermocycler (PE Biosystems, https://www.thermofisher.com) and analyzed the DNA fragments using 2% agarose gel electrophoresis. The expected PCR fragments from primary PCR were 1,481 bp and from secondary PCR were 317–320 bp.

Appendix Table 1. Nested PCR detection of *Plasmodium cynomolgi*

Primers	Sequence (5′ → 3′)	Positions after the RO strain*
	Primary PCR	
PCOX1-F0	CTTTTAACGCCTGACATGGATGGATAATACTCG	3,196–3,228
PCOX1-R0	TCTGGATAATCAGGAATACGTCTAGGCATTAC	4,645–4,676
	Secondary PCR	
Pcy1COXI-F	CCAAGCCTCACTTATTGTTAATTTATTTTT	3,291–3,320
Pcy2COXI-F	CTTATTGTTAATTATATATTGTATTATATATTTTTTG	†
PcyCOXI-R	CTGGAGAACCACATAAAATTGGTAAAAAA	3,579–3,607

*GenBank accession number AB444131
†Sequence after *P. cynomolgi* from macaques in Thailand not found in the sequence of the RO strain (Putaporntip et al., unpub. data)

Appendix Table 2. Pairwise sequence comparison of the mitochondrial cytochrome oxidase I genes of human and simian malaria species*†

Strain	*P. cynomolgi* RO strain AB444131	*P. cynomolgi* Gombak strain AB444129	*P. fieldi* AB444132	*P. simiovale* AB434920	*P. vivax* AY791544	*P. fragile* AB444135	*P. knowlesi* AY598141	*P. hylobati* AB354573	*P. coatneyi* AB354575	*P. inui* AB444114	*P. gonderi* AB434918	*P. malariae* AB354570	*P. ovale* AB354571	*P. falciparum* AJ276845
P. cynomolgi RO strain AB444131	NA	12	15	16	21	39	47	54	55	62	80	105	103	172
P. cynomolgi Gombak strain AB444129	99.09	NA	16	19	21	36	33	51	49	36	83	112	107	180
P. fieldi AB444132	98.79	98.86	NA	16	23	35	37	42	47	57	80	116	110	184
P. simiovale AB434920	98.79	98.56	98.79	NA	19	38	34	43	45	58	78	112	110	182
P. vivax AY791544	98.56	98.25	98.41	98.41	NA	31	39	42	44	44	76	106	114	180
P. fragile AB444135	97.65	97.12	97.34	97.27	97.04	NA	33	38	40	44	77	109	113	179
P. knowlesi AY598141	97.50	97.04	97.42	97.19	97.50	96.43	NA	34	40	45	75	104	112	180
P. hylobati AB354573	97.42	97.12	96.81	96.74	96.81	96.13	95.90	NA	38	43	65	109	108	180
P. coatneyi AB354575	97.12	96.97	96.97	96.66	96.59	96.43	96.28	95.83	NA	38	66	102	104	176
P. inui AB444114	97.12	96.74	96.59	96.66	96.66	95.60	95.68	97.27	95.30	NA	64	106	96	174
P. gonderi AB434918	95.14	94.99	95.07	94.31	94.16	94.23	94.08	93.93	93.70	93.93	NA	103	101	177
P. malariae AB354570	92.19	91.96	92.26	91.73	92.11	91.73	91.96	91.50	91.20	91.50	92.03	NA	104	174
P. ovale AB354571	92.11	92.34	92.72	92.11	91.81	91.50	91.43	91.35	91.65	91.65	91.88	92.19	NA	176
P. falciparum AJ276845	86.65	86.80	86.57	86.80	86.65	86.34	86.34	86.42	86.34	86.19	86.04	86.34	86.95	NA

*NA, not applicable.

†Homology inferred from percentage sequence identity are shown in the lower left corner cells and the numbers of pairwise nucleotide differences in the upper right corner cells. Sequences contain 1,318 bp. GenBank accession numbers are shown below the species names.